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Role of CXCR5 and CCR7 in Follicular Th Cell Positioning and Appearance of a Programmed Cell Death Gene-1\textsuperscript{High} Germinal Center-Associated Subpopulation\textsuperscript{1}

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Th cell access to primary B cell follicles is dependent on CXCR5. However, whether CXCR5 induction on T cells is sufficient in determining their follicular positioning has been unclear. In this study, we find that transgenic CXCR5 overexpression is not sufficient to promote follicular entry of naive T cells unless the counterbalancing influence of CCR7 ligands is removed. In contrast, the positioning of Ag-engaged T cells at the B/T boundary could occur in the absence of CXCR5. The germinal center (GC) response was 2-fold reduced when T cells lacked CXCR5, although these T cells were able to access the GC. Finally, CXCR5\textsuperscript{high}CCR7\textsuperscript{low} T cells were found to have elevated IL-4 transcript and programmed cell death gene-1 (PD-1) expression, and PD-1\textsuperscript{high} cells were reduced in the absence of T cell CXCR5 or in mice compromised in GC formation. Overall, these findings provide further understanding of how the changes in CXCR5 and CCR7 expression regulate Th cell positioning during Ab responses, and they suggest that development and/or maintenance of a PD-1\textsuperscript{high} follicular Th cell subset is dependent on appropriate interaction with GC B cells. The Journal of Immunology, 2007, 179: 5099–5108.

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\textsuperscript{6}Abbreviations used in this paper: GC, germinal center; ASC, Ab-secreting cell; DEL, duck egg lysozyme; FDC, follicular dendritic cell; HEL, hen egg lysozyme; int, intermediate; NP, (4-hydroxy-3-nitrophenyl)acetyl; CCG, chicken γ-globulin; PD-1, programmed cell death gene-1; SDF-1, stromal cell-derived factor.

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B cell homing into follicles is dependent on the follicular chemokine, CXCL13 (BLC, BCA1), and its receptor, CXCR5, which is expressed by all mature B cells (1, 2). Within 3 days of Ag recognition and activation in the T zone, CD4 T cells can also find within B cell follicles (3, 4). Th cells begin up-regulating CXCR5 within a few days of Ag recognition, achieving maximal levels within 5 days, whereas the function of CCR7, the receptor for the T zone chemokines CCL19 (ELC) and CCL21 (SLC), is concordantly reduced (5, 6). Indeed, analysis of T cells isolated from human peripheral blood has demonstrated that many CXCR5-expressing T cells have down-regulated CCR7 expression levels (7, 8). In two recent studies, it was demonstrated that CXCR5 expression on T cells is necessary for their access into B cell follicles (9, 10). However, whether CXCR5 induction was sufficient for follicular homing of T cells during an Ab response was not directly tested in these experiments.

T cells isolated from lymphoid tissue based on CXCR5 expression have been found to possess potent B cell helper activity (6, 8, 11, 12). In addition to providing help for B cell proliferation, plasma cell formation, and isotype switching, T cells are essential for the development and maintenance of the germinal center (GC)\textsuperscript{6} response (13). GC-associated T cells are typically enriched within the T zone distal pole of the GC, known as the light zone, and they express high levels of CXCR5 (8, 11, 12). The costimulatory molecule, ICOS, is also abundantly expressed on follicular Th cells (14, 15). Recent gene expression array studies identified additional genes that are up-regulated in CXCR5\textsuperscript{+} T cells (7, 16, 17), including programmed cell death gene-1 (PD-1), a negative regulator of T cell activation (18). Immunohistochemical analysis of human tonsil tissue has shown that PD-1\textsuperscript{+} T cells are enriched within the GC light zone (19), although the distribution of these cells in mouse lymphoid tissues has not been reported.

To determine whether CXCR5 was both necessary and sufficient for T cell migration into follicles, we examined the migratory activity of adoptively transferred CXCR5-deficient and CXCR5-overexpressing T cells in wild-type and CCR7 ligand-deficient mice. We also tested whether CCR7 down-modulation was important by examining T cell behavior in CCR7 transgenic mice. Our findings confirm the importance of CXCR5 in T cell homing to follicles and further define how the shift in CXCR5 and CCR7 expression controls Th cell positioning. We also identify an important role for T cell CXCR5 in supporting GC responses of normal magnitude. In addition to this, our experiments reveal a requirement for T cell CXCR5 to allow the normal accumulation of a PD-1\textsuperscript{high} GC-associated T cell population.

Materials and Methods
Mice and chimeras
Six- to 8-wk-old C57BL/6J (B6) mice, B6-µMT (Ig-6m<sup>con</sup>) mice, and B6-TCR<sup>β</sup>δ<sup>−/−</sup> (TCR<sup>β</sup>δ<sup>−/−/α</sup>δ<sup>−/−</sup>) mice were obtained from The Jackson Laboratory. Six- to 8-wk-old 129S6-RAG2-M (Rag2<sup>2m/2m</sup>) mice were obtained from Taconic Farms. OVA-specific TCR transgenic mice on the B6 background were of the OT-II line (20). These mice were crossed to C57BL/6J (B6) mice on a B6 background. VDJ<sub>H</sub> HEL10 H chain knockin and CD<sub>5</sub> L chain transgenic mice were as described (21). B6-4<sup>gt</sup> reporter mice (22) were provided by R. Locksley (University of California, San Francisco, CA). These mice were crossed to both the OT-II wild-type and CCRX<sup>5/−</sup> B6 lines. CCR7 transgenic mice were on the B6 background (23). These mice were used to generate an OT-II CCR7 transgenic line. CD19<sup>−/−</sup> mice (24) on the B6 background were provided by K. Rajwesky (Harvard Medical School, Boston, MA). These mice, along with the B6-µMT mice, B6-TCRδ<sup>−/−</sup> mice, and 129S6-RAG2-M mice, were maintained and bred in the University of California transgenic barrier under the approval of the Institutional Animal Care and Use Committee.

Generation of transgenic mice
CCR5 transgenic mice were produced by inserting the full-length cDNA of mouse CCR5 with a prolactin-flag sequence at the 5' end (27) into the BamHI site of the plasmid p1026x containing the lck promotor interrupted by the Eμ H chain enhancer (28). Incorporated into the 3' end of the plasmid was a mutated human growth hormone sequence containing an intron and poly(A) site (28). Pronucleot mediated of the linearized lck/lk<sub>Eμ</sub>-CCR5 minigene, lacking the vector sequences, into fertilized B6.D2F1 oocytes was performed according to standard procedures. Two founder mice (µ29 and µ30; referred to in this work as X<sup>5/5</sup> and X<sup>3/3</sup>, respectively) were selected that contained circulating CCR5-expressing naïve T cells. The mice used in these studies had been backcrossed for at least 10 generations to the B6 background.

Transfers and immunizations
Adoptive T/B cotransfers and immunization of recipients were conducted, as previously described (21). Lymphocytes were isolated from the peripheral lymph nodes (LN) and/or spleen of donor mice by mechanical disruption on 70-µm nylon cell strainers (Falcon) in DMEM (Mediatech). The frequency of unpurified TCR transgenic CD<sub>4</sub> T cells isolated from donor CCRX<sup>5/−</sup> or CCRX<sup>5/−/OT-II</sup> CD4<sub>5</sub>1 mice (V<sub>α2</sub> V<sub>B5</sub> T cells) and hen egg lysozyme (HEL)-binding B cells isolated from donor CDV9/Sl5 mice was determined by flow cytometric analysis. TCR transgenic OT-II T cells (1.0 × 10<sup>5</sup>–3.3 × 10<sup>5</sup>) and HEL-binding VDJ9/Sl6 B cells (3.0 × 10<sup>3</sup>–1.0 × 10<sup>4</sup>) were cotransferred by i.v. injection into the lateral tail vein of sex-matched C57BL6/10 recipients. Twenty-four hours post-T/B cell transfer; recipient mice were immunized s.c. across six sites (neck, shoulders, and right and left flank) with a total of 50 µg of a duck egg lysozyme (DEL)-OVA conjugate in alumin. Draining axillary, brachial, and inguinal nodes were isolated 6–7 days postimmunization for analysis. In some positional experiments, only wild-type, CCRX<sup>5/−</sup>, or CCR7 transgenic OT-II T cells (5 × 10<sup>5</sup>) were transferred i.v. into naïve B6 recipients before immunization. Twenty-four hours post immunization, mice were immunized s.c. over six sites with a total of 50 µg of OVA, emulsified in CFA. This immunization protocol was also used in positional studies conducted in CCR7 transgenic mice. Draining LN were harvested 5 days after immunization.

For T cell reconstitution of TCRδ<sup>−/−</sup>δ<sup>−/−</sup> mice, 1.5 × 10<sup>7</sup> CCRX<sup>5/−</sup> or CCRX<sup>5/−/OT-II</sup> nontransgenic T cells were injected i.v. After 5 days, the mice were immunized i.p. with SRBC (Colorado Serum) as described (25), or 100 µg total of (4-hydroxy-3-nitrophenyl)acetyl (NP<sub>4</sub>chicken H<sub>2</sub>K<sub>B</sub>) (NP<sub>4</sub>-chicken H<sub>2</sub>K<sub>B</sub>) (Biosearch Technologies) in alumin. Mice were sacrificed at day 8 (SRBC) or days 6 and 14 (NP-CGG) after immunization. A similar protocol was used for the immunization and analysis of experiments involving bone marrow chimeras, CCR7 transgenic mice, and CD19-deficient mice.

CXCR5-mediated positional studies involved enriching CD4<sub>+</sub> T cells from the 10<sup>7</sup> cell suspension (µ29 X<sup>5/5</sup> and µ30 X<sup>3/3</sup>) mice and cell-mixing the cell suspension by autoMACS (Miltenyi Biotec) purification. For all transfers, 10<sup>7</sup> cells were labeled with CFSE (Molecular Probes), as described (29), and transferred by i.v. injection into the lateral tail vein of sex-matched B6 wild-type or RAG1<sup>−/−</sup> recipient mice. After 48 h, recipients were sacrificed and spleen sections were frozen in OCT for sectioning.

Immunohistochemistry, flow cytometry, chemotaxis, and ELISPOT
For immunohistochemistry, cryostat sections (7 µm) were acetone fixed and stained (as described previously (25), with mixtures of two of the following reagents: sheep anti-IgG (purified; The Binding Site), rat anti-IgD (11-26.2a, purified; BD Pharmingen), rat anti-CD35/CR1 (8C12, purified; BD Pharmingen), Armenian hamster anti-CD3e (145-2C11, purified; BD Pharmingen), rat anti-follicular dendritic cell (FDC) (FDC-M2, biotinylated; ImmunoKontact), rat anti-CXCR4 (2B11/2/CXCR4, biotinylated; BD Pharmingen), anti-CD16/sCD15 (FcγRIII) (2.4G2, FITC-conjugated; BD Pharmingen), and rat anti-PD-1 (J43, purified; eBioscience). Biotinylated reagents were detected with streptavidin-alkaline phosphatase (Jackson ImmunoResearch Laboratories), and rat and Armenian hamster mAbs were detected with HRP-conjugated donkey anti-rat IgG (H + L) or goat anti-Armenian hamster IgG (H + L) conjugates (Jackson ImmunoResearch Laboratories), respectively. FITC-conjugated primary Abs and CFSE-labeled cells were detected with sheep anti-fluorescein-alkaline phosphatase Fab (anti-fluorescein AP; Roche). Enzyme reactions were developed with conventional substrates for peroxidases (diaminobenzidine/H<sub>2</sub>O<sub>2</sub>Sigma-Aldrich) and alkaline phosphatase (FastBlue/Naphthol AS-MX, Sigma-Aldrich). Endogenous alkaline phosphatase activity was blocked with levamisole (Sigma-Aldrich). Sections were viewed on a Leica DMRL fluorescence microscope.

For flow cytometric analysis, spleen and/or LN suspensions were stained, as previously described (21). Data were collected on a FACSCalibur (BD Immunocytometry Systems) or LSRII (BD Immunocytometry Systems) and analyzed with FlowJo software (Tree Star). The following anti-mouse mAbs were purchased from BD Pharmingen (unless otherwise indicated): anti-CD4 (FITC, Caltag Laboratories; PE-Cy7, Alexa Fluor 700, BD Pharmingen), anti-CD45.1/II/III (2.4G2, FITC-conjugated; BD Pharmingen), and anti-PD-1 (J43, purified; eBioscience), biotinylated anti-
ICOS (eBioscience), and anti-PD-1-PE (eBioscience). CCR7 was stained with CCL19-Fc, as described (30), using a biotin-conjugated goat anti-human IgG, Fc fragment-specific, detection Ab (Jackson ImmunoResearch Laboratories). CXCR5 was stained with a purified anti-CXCR5 mAb (BD Pharmingen) before the addition of a biotinylated anti-rat IgG (H+L) mAb (Jackson ImmunoResearch Laboratories) that had been preabsorbed with 4% normal mouse serum (Sigma-Aldrich). Alternatively, an anti-rat allophycocyanin reagent was used (Jackson ImmunoResearch Laboratories). A similar approach was used when staining for PD-1 using the purified anti-PD-1 mAb (J43; eBioscience). Biotinylated Abs were detected with streptavidin allophycocyanin or Qdot 605 (Invitrogen Life Technologies). For the enumeration of HEL-binding B cells, cell suspensions were first incubated with 1 μg/ml HEL (Sigma-Aldrich), washed, and then stained with HyHEL9 PE-Cy5.5 (custom conjugated by Caltag Laboratories). For cell analysis by flow cytometry, cells were resuspended in PBS containing 2% FBS and 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich) at a final concentration of 100 ng/ml.

Chemotaxis assays were performed, as described (25), using 5 μM transwells (Corning Costar). Transmigrated cells were enumerated by flow cytometry. Duplicate wells were analyzed for each concentration of chemokine. Mouse rCXCL13 was from R&D Systems, and human recombinant stromal cell-derived factor (SDF-1) was from PeproTech. ELISPOT assays were performed, as described (31).

Statistical analysis
A nonparametric two-tailed Mann-Whitney U test was used to determine statistical significance.

FIGURE 2. CXCR5 overexpression on T cells augments their chemotaxis to CXCL13 and promotes migration into follicles when CCR7 ligands are deficient. A, Flow cytometric analysis of CXCR5 expression levels on naïve CD4 T cells from wild-type mice (naive WT) and μ30 (X5low) and μ29 (X5high) CXCR5 transgenic mice. For comparison, the CXCR5 levels on activated OT-II TCR transgenic T cells 5 days postimmunization (Activated OT-II) and naïve nontransgenic B cells are shown. B, Transwell migration of naïve splenic CD4 T cells from the X5high (○) and X5low (□) transgenic mice and naïve CD4 T cells (WT, ▲) and B cells (■) from control littersmates to the specified concentrations of CXCL13 (BLC) or CCL21 (SLC). Data are representative of three individual experiments; n = 6. Error bars, ± SD. C, Immunohistochemistry of spleen tissue from wild-type or plt/plt mice that had received CFSE-labeled CD4 T cells from wild-type (WT) or X5high or X5low transgenic mice 48 h earlier. CFSE-labeled cells were identified with anti-FITC (blue), and B cell areas were delineated with anti-IgD (brown). T, T zone; F, follicle. Objective magnification, ×5. Data are representative of seven experiments.

FIGURE 3. Down-modulation of CCR7 is required for T cell homing to follicles. A, Flow cytometric analysis of CCR7 (left panel) and CXCR5 (right panel) expression on CCR7 transgenic (CCR7Tg, solid line) and wild-type (WT, dashed line) CD44highCD62Llow OT-II CD4 T cells from peripheral LN, isolated 5 days post-s.c. immunization with OVA in CFA. For comparison, the CCR7 and CXCR5 levels on CD44highCD62Lhigh OT-II CD4 T cells (Naive WT, solid histogram) are shown. B, Immunohistochemistry on cryostat sections of LN from immunized wild-type (left panel) or CCR7 transgenic (right panel) mice, stained to detect total T cells (CD3, blue) and B cells (IgD, brown). C, Immunohistochemistry on cryostat sections of LN isolated from immunized wild-type mice that had received wild-type (left panel) or CCR7 transgenic (right panel) OT-II T cells. Sections were stained to detect transferred T cells (CD45.1, blue) and endogenous B cells (IgD, brown). In both systems, LN were harvested 5 days postimmunization. T, T zone; F, follicle. Objective magnification, ×5. Data are representative of three peripheral LN isolated from six and three mice, respectively, from three separate experiments.
Results

CXCR5 induction is necessary, but not sufficient for T cell follicular homing

We first tested whether CXCR5 was required for follicular homing of activated T cells by transferring CXCR5-deficient or wild-type OT-II TCR transgenic T cells and were immunized s.c. with DEL-OVA in alum. LN were harvested 7 days postimmunization. Data are representative of four experiments (n = 12 mice). Bars show mean ± SD. B. Flow cytometric analysis of CCR7 levels on wild-type (X5+/−) and CXCR5−/− OT-II T cells, determined using CCL19-Fc, at day 7 postimmunization. For comparison, CCR7 levels on endogenous Vβ5+ CD4 T cells are shown (Naive, solid histogram). C. Total numbers of HEL-specific ASC of the indicated isotopes in the draining LN of the mice described in A, quantitated by ELISPOT. Generation of ASC was not detected in the peripheral LN of mice that had received only HEL-binding VDJ9/ε5 B cells, but had been subjected to the same immunization regime (data not shown). D. Comparison of the total number of HEL-specific VDJ9/ε5 B cells (left panel) and GC B cells (GL7+ IgD−/− Fas−/− CD19+) in the draining LN of the mice described in A. Expansion of HEL-specific B cells and GC B cells in mice that received only HEL-binding VDJ9/ε5 B cells, but had been subjected to the same immunization regime was also examined (B alone; n = 3). Background expansion of endogenous HEL-binding B cells in immunized wild-type mice that received no T or B cells is also shown (no transfer; n = 3). Bars, Mean ± SD; *, p = 0.004. E. Lack of transferred CXCR5−/− OT-II T cells and presence of endogenous T cells in DEL-OVA-induced GC. Immunohistochemistry of cryostat sections of LN from mice that had received VDJ9/ε5 and wild-type or CXCR5-deficient OT-II T cells, as indicated, at day 7 after immunization with DEL-OVA in alum. Sections were stained for IgD (brown) and CD45.1 (blue) to identify the transferred OT-II T cells or anti-CD3ε (blue) to locate all T cells. Objective magnification, ×5. Data are representative of LN from more than three mice of each type.

FIGURE 4. T cell CXCR5 is not required to support early T-B cell collaboration and plasma cell formation, but is necessary for normal GC development. A. Quantitation of the total number of wild-type and CXCR5−/− OT-II transgenic T cells in the draining axillary, brachial, and inguinal LN. Groups of three mice received a 3:1 mix of HEL-specific VDJ9/ε5 B cells and OT-II T cells and were immunized s.c. with DEL-OVA in alum. LN were harvested 7 days postimmunization. Data are representative of four experiments (n = 12 mice). Bars show mean ± SD. B. Flow cytometric analysis of CCR7 levels on wild-type (X5+/−) and CXCR5−/− OT-II T cells, determined using CCL19-Fc, at day 7 postimmunization. For comparison, CCR7 levels on endogenous Vβ5+ CD4 T cells are shown (Naive, solid histogram). C. Total numbers of HEL-specific ASC of the indicated isotopes in the draining LN of the mice described in A, quantitated by ELISPOT. Generation of ASC was not detected in the peripheral LN of mice that had received only HEL-binding VDJ9/ε5 B cells, but had been subjected to the same immunization regime (data not shown). D. Comparison of the total number of HEL-specific VDJ9/ε5 B cells (left panel) and GC B cells (GL7+ IgD−/− Fas−/− CD19+) in the draining LN of the mice described in A. Expansion of HEL-specific B cells and GC B cells in mice that received only HEL-binding VDJ9/ε5 B cells, but had been subjected to the same immunization regime was also examined (B alone; n = 3). Background expansion of endogenous HEL-binding B cells in immunized wild-type mice that received no T or B cells is also shown (no transfer; n = 3). Bars, Mean ± SD; *, p = 0.004. E. Lack of transferred CXCR5−/− OT-II T cells and presence of endogenous T cells in DEL-OVA-induced GC. Immunohistochemistry of cryostat sections of LN from mice that had received VDJ9/ε5 and wild-type or CXCR5-deficient OT-II T cells, as indicated, at day 7 after immunization with DEL-OVA in alum. Sections were stained for IgD (brown) and CD45.1 (blue) to identify the transferred OT-II T cells or anti-CD3ε (blue) to locate all T cells. Objective magnification, ×5. Data are representative of LN from more than three mice of each type.

CXCR5 induction is necessary, but not sufficient for T cell follicular homing

We first tested whether CXCR5 was required for follicular homing of activated T cells by transferring CXCR5-deficient or wild-type OT-II TCR transgenic T cells into wild-type recipients and immunizing with OVA in adjuvant. Five days later, a time point when the cells have undergone considerable proliferation and have upregulated CXCR5 (5, 32) (data not shown), numerous wild-type OT-II T cells were detectable within IgD−/−H11001 follicles (Fig. 1). By contrast, CXCR5-deficient cells failed to gain access to the follicular compartment (Fig. 1). Similar findings were recently reported by others using different TCR transgenic systems (9, 10). Occasional CXCR5-deficient T cells were found at the rear of follicles in a subcapsular location, but few, if any, cells were found overlapping with the CD35+ FDC network.

To examine whether CXCR5 up-regulation was sufficient for T cell migration into follicles, we tested the effect of transgenically expressing CXCR5 in naive CD4 T cells. Two transgenic mouse lines were generated that expressed CXCR5 under control of the lck-proximal promoter and Ig H chain enhancer. In the CXCR5 transgenic line, μ30 (referred to as X5+/+), naive CD4 T cells expressed receptor levels similar to day 5 activated nontransgenic CD4 T cells, whereas in the line μ29 (referred to as X5+/-), naive CD4 T cell expression of CXCR5 was almost equivalent to that on B cells and several-fold higher than on activated T cells (Fig. 2A).

In chemotaxis assays, naive T cells from both CXCR5 transgenic lines responded to CXCL13 with the magnitude of the response...
Maintenance of CCR7 expression on activated T cells impedes follicular entry

To determine whether CCR7 down-regulation was the principal mechanism acting to reduce the function of CCR7 in activated Th cells, we examined the effect of forcibly maintaining CCR7 expression levels. For this we employed the use of CCR7 transgenic mice, in which the expression of the chemokine receptor was controlled by regulatory elements from the Cd4 minigene (23). The expression of CCR7 on naive CD4 T cells in the transgenic mice is only slightly higher than on naive CD4 T cells from wild-type controls (23). At day 5 following immunization with Ag in adjuvant, many of the activated CD44highCD62Llow cells had reduced CCR7 surface expression (Fig. 3A), as expected (37); in the CCR7 transgenic mice, most of these cells continued to express CCR7 at levels similar to the starting amounts on naive T cells (Fig. 3A). Up-regulation of CCR5 occurred equivalently on CCR7 transgenic and wild-type CD44highCD62Llow cells (Fig. 3A). Analysis of tissue sections showed that few CCR7 transgenic T cells gained access to lymphoid follicles in contrast to the efficient access observed for wild-type cells (Fig. 3B). Similarly, transferred CCR7 transgenic OT-II T cells at day 5 of the response to Ag in adjuvant showed a marked defect in their appearance within lymphoid follicles compared with control OT-II T cells (Fig. 3C). These findings strongly suggest that CCR7 down-regulation is a key mechanism acting in activated CD4 T cells to overcome responsiveness to T zone chemokines and retention in the T zone.

FIGURE 5. CXCR5-deficient T cells access GC, but fail to accumulate normally in light zones. A. Quantitation of the total number of GC B cells (GL7+IgD–/–Fas–/–CD19+) present in the spleens of immunized TCRβ+/–δ−/– mice to which CXCR5-deficient or wild-type nontransgenic T cells had been transferred. Mice were immunized i.p. with SRBC or NPα/CGG in alum and analyzed 8 or 14 days later, respectively. Each point indicates an individual animal, and bars represent the mean. B. Immunohistochemistry on spleen sections from NP-CGG/alum-immunized TCRβ+/–δ−/– mice reconstituted with wild-type or CXCR5–/– T cells. Spleens were harvested at day 14 after immunization. Sections were stained with GL7 (blue) to detect GC B cells, together with anti-IgD (brown) to delineate the B cell zones. Data are representative of spleen sections from three mice. Objective magnification, ×5. C. Immunohistochemistry of GC within the splenic white pulp of T cell-reconstituted TCRβ+/–δ−/– mice at day 14 after immunization with NPα/CGG in alum. Serial cryostat sections were stained with anti-IgD (brown) to define the surrounding follicular mantle zone (FM) together with anti-FcγRII/III (blue; upper panel) or anti–CXCR4 (blue; middle panel) to reveal the light and dark zone boundaries (dashed lines), respectively. Consecutive sections were stained with corresponding to the CXCR5 expression level, whereas naive nontransgenic cells were unresponsive (Fig. 2B). In addition, as previously observed for activated T cells (5), the CXCR5 transgenic T cells responded chemotactically to lower doses of chemokine than is typically observed with B cells (Fig. 2B). This difference in dose sensitivity therefore appears to reflect intrinsic differences between T and B cells rather than being due to the activation status of the cells.

Upon transfer into wild-type recipients, the CXCR5 transgenic T cells localized to the outer regions of the T zone, but they failed to become dispersed within follicles (Fig. 2C). Notably, T cells from line μ29 (X5high) displayed a tendency to enter the T zone proximal region of the follicle, a zone that has been shown to contain a decreasing gradient of the CCR7 ligand, CCL21 (33). Indeed, previous work has indicated that B cell access to this zone is in part CCR7 ligand dependent (33, 34). To determine whether the CXCR5-expressing T cells were being retained at the follicular boundary by T zone chemokines, transgenic or control cells were transferred into CCR7 ligand-deficient plt/plt recipients (35, 36). In these animals, the X5high transgenic cells became uniformly distributed within follicles, whereas wild-type T cells were unable to enter these compartments (Fig. 2C). The X5low cells showed an intermediate phenotype, with the cells entering the outer regions of the follicles, but not becoming uniformly distributed (Fig. 2C). These findings establish that CXCR5 up-regulation is not sufficient for a CD4 T cell to access lymphoid follicles, and they indicate that additional changes, such as reduced responsiveness to CCR7 ligands, must also occur.
CXCR5 is not essential for day 7 T cell-mediated Ab responses

To examine the impact of T cell CXCR5 deficiency on the Ab response, CXCR5<sup>−/−</sup> or wild-type OT-II T cells together with VDJ9/κ5 lysozyme-specific B cells (21) were cotransferred into syngeneic recipients that were then immunized with DEL-OVA. Analysis 7 days later revealed that both wild-type and CXCR5<sup>−/−</sup> OT-II T cells had undergone similar levels of expansion (Fig. 4A) and the reduction in CCR7 surface abundance was similar between the two groups (Fig. 4B). Furthermore, similar numbers of HEL-specific IgM and IgG2a Ab-secreting cells (ASC) were induced (Fig. 4C). Early B/T interactions between Ag-specific B and T cells and the first appearance of ASC are thought to occur at the B/T zone boundary (32, 38, 39). Analysis of draining LN at day 7 revealed a similar distribution of wild-type and CXCR5-deficient OT-II T cells in the T zone and along the B/T boundary region (Fig. 4E, and data not shown). These findings suggest that CXCR5 is not essential for CD4 T cells to localize to the B/T boundary to support induction of plasma cell responses.

T cell CXCR5 is required for a GC response of normal magnitude

In the cotransfer system discussed above, the magnitude of the GC response was ~2-fold reduced when the OT-II cells were CXCR5 deficient (Fig. 4D). Because the total number of Ag-specific T cells was not affected by CXCR5 deficiency (Fig. 4A), these findings suggested a selective defect in the ability of the CD4 T cells to support the GC response. However, assessment of T cell distribution within GC was confounded by the finding that in recipients of CXCR5<sup>−/−</sup> OT-II T cells, endogenous wild-type T cells began participating in the GC response by day 7 (Fig. 4E). To further examine the impact of CXCR5 deficiency in T cells on GC responses, we reconstituted T cell-deficient mice with either wild-type or CXCR5-deficient T cells and then immunized with SRBC or NP<sub>25</sub>-CGG in alum. The responses to these distinct immunogens had different time courses, but as in the transgenic T/B co-transfer experiments, similar IgM and IgG1 plasma cell numbers were detected in both the wild-type and knockout groups (data not shown). Importantly, the GC responses were reduced ~2-fold in mice bearing CXCR5<sup>−/−</sup> T cells (Fig. 5A). Immunohistochemical analysis confirmed that the GC that formed in these animals were typically smaller than those seen in the wild-type groups (Fig. 5B). GC that were supported by CXCR5<sup>−/−</sup> T cells developed in a wild-type location within follicles, and normal dark and light zone polarity was established, as determined by staining for the light zone FDC markers, FcγRIIB (40) (Fig. 5C) and FDC-M2 (25) (data not shown), and for the dark zone with an anti-CXCR4 Ab (Fig. 5C). In contrast to the near-complete block of CXCR5-deficient T cell access to follicles, staining for CD3 revealed the presence of CXCR5-deficient T cells in GC, although at a lower frequency than that detected in wild-type GC (Fig. 5, C and D). Although Ag-reactive T cells are typically enriched within the light zone pole of the GC (13), GC-associated CXCR5<sup>−/−</sup> T cells failed to become enriched within the FcγRIIB<sup>+</sup> light zone (Fig. 5, C and D), suggesting that CXCR5 is involved in coordinating appropriate T cell positioning within the GC microenvironment. Similar observations were made in bone marrow chimeric mice that had been reconstituted with CXCR5<sup>−/−</sup> T cells and wild-type B cells (data not shown; see Materials and Methods). A further series of SRBC immunization experiments were performed in the CCR7 transgenic mice (Fig. 6). As expected, the CD44<sup>high</sup>/CD62L<sup>low</sup> CD4 T cells induced in these mice following immunization did not down-regulate CCR7 to the extent observed for wild-type cells (Fig. 6A, upper panel), whereas CXCR5 was up-regulated normally (Fig. 6A, lower panel). This selective inability of activated T cells to down-regulate CCR7 was associated with a reduced GC response, reflected both in the size of the GC (Fig. 6B) and in the
FIGURE 7. Development of a PD-1high Th cell subset is dependent on T-B cell interactions and GC formation. A. Flow cytometric analysis of transferred OT-II T cells in mice that received a mixture of VDJ9α5 B cells and OT-II T cells and were immunized s.c. with DEL-OVA in alum. By day 7 of the immune response, wild-type OT-II T cells (identified as Vβ5+ Vα2+ CD4+ CD45.1+ cells) could be divided into three subsets differing in CXCR5 and CCR7 expression levels (gates I-III). The frequency of OT-II T cells in each of the gates is shown. Histogram plots show the expression of ICOS, PD-1, and IL-4 transcripts (revealed with a GFP reporter) in the three gated T cell subsets and are representative of data from 12 mice. B. Immunohistochemistry of serial cryostat sections showing the location of PD-1+ T cells within the splenic white pulp of mice at day 8 of the immune response to SRBC.
total number of GC B cells that could be identified by flow cytometry (Fig. 6C). Thus, CXCR5 up-regulation and CCR7 down-regulation both appear to be required for Th cells to support a GC response of normal magnitude.

**CXCR5- and B cell-dependent development of a PD-1high Th cell subset**

In an effort to quantitate GC-associated T cells by flow cytometry, we examined Ag-engaged T cells for their expression of CXCR5, CCR7, ICOS, and PD-1. Because IL-4 has been reported in some studies to contribute to the GC response (41, 42), we also examined IL-4 transcript levels in CXCR5+CD4+ T cells by intercrossing the TCR transgenic mice with 4get reporter mice (22) that express GFP from the IL-4 locus. In our OT-II-VDJ9/α5 cotransfer system, all detectable OT-II T cells were found to express similar levels of ICOS (Fig. 7A). By contrast, PD-1 expression levels and the abundance of IL-4 transcripts were greatest in the CXCR5highCCR7low subset of OT-II T cells (Fig. 7A). Based on these flow cytometric analyses, we speculated that within this subset might reside a GC-associated CD4+ T cell population. Indeed, PD-1 has been shown to be abundantly expressed by GC-associated T cells in human tonsils (19, 43). We found in mouse spleen that a large majority of the intensely PD-1-staining T cells were GC associated, although some of these cells were also detected in the follicular mantle surrounding the GC (Fig. 7B). In the GC that emerge spontaneously in Peyer’s patches, PD-1high T cells were abundant and largely restricted to the GC light zone (Fig. 7C). However, it should be noted that GC-associated T cells are heterogeneous in their intensity of staining for PD-1, suggesting that the PD-1int-low T cell subsets may also participate in the GC response (Fig. 7C, and data not shown). Based on these observations, we next tested whether CXCR5 deficiency affected the frequency of PD-1high T cells; because we could not track the cells based on CXCR5, we instead gated on CCR7low cells. In the cotransfer system involving CXCR5-deficient OT-II T cells, PD-1highCCR7low T cells were almost completely absent (Fig. 7D). Because we had found that by day 7 the GC contained endogenous T cells and few transferred CD45.1 CXCR5+/- OT-II T cells (Fig. 4E), this suggested that the presence of PD-1high T cells did not depend on GC association. Indeed, immunohistochemical analysis of CXCR5+/- T cells in the spleens of reconstituted TCRβ+/-δ+/- mice revealed that PD-1high, CXCR5-deficient T cells did develop and reside within GC, but at a lower frequency than that seen in mice bearing wild-type T cells (data not shown). These data also suggested that the marked deficiency of PD-1high cells in the CXCR5+/- OT-II plus VDJ9/α5 cotransfer system was due to competition with endogenous wild-type T cells. To further examine the relationship between PD-1 induction and T cell interaction with B cells, we performed OT-II T cell transfers into μMT mice that lack B cells. In these mice, the PD-1high CD4 T cell population, identified as PD-1highCXCR5high or PD-1highCCR7low, failed to accumulate within the draining LN following immunization (Fig. 7E). Further to this, we performed OT-II T cell transfers into CD19-deficient mice that contain normal B and T cell numbers and are able to support early plasma cell responses, but are defective in mounting GC responses (24). Few PD-1highCXCR5high or PD-1highCCR7low CD4 T cells developed in the spleens of CD19-deficient mice following immunization (Fig. 7E). Thus, these findings indicate that PD-1high CD4 T cells within lymphoid organs are enriched for GC T cells, and they suggest that the appearance of these cells is a result of persistent interactions with Ag-presenting GC B cells.

**Discussion**

The above findings demonstrate that CXCR5 up-regulation is not sufficient for Th cell migration into follicles; T cells must also down-regulate CCR7 to achieve a sufficient change in the balance of chemokine responsiveness to favor migration into the CXCL13-positive follicles. We also find that T cell expression of CXCR5 is necessary for mounting a GC response of normal magnitude. However, in contrast to the requirement for follicular access, CXCR5-deficient T cells can access the GC, indicating that additional cues are involved in directing T cells to this compartment. Finally, we provide evidence that the appearance or accumulation of ICOS+ T cells expressing high amounts of PD-1 depends on interaction with GC B cells. We speculate that chronic exposure to Ag-presenting B cells in the GC contributes to the maintained expression of PD-1 on GC-associated T cells.

The CXCR5 requirement for T cell access to follicles has recently been demonstrated in transfer experiments with CXCR5-deficient TCR transgenic T cells (9, 10). In one of these studies, activated CCR7-deficient T cells were suggested to more readily access follicles (9). These experiments did not exclude the possibility that additional changes must occur during T cell activation to allow T cell access to follicles. By examining the behavior of naive T cells that have been selectively modified to express CXCR5, we show that CXCR5 up-regulation is not sufficient to permit T cell localization within B cell follicles. However, removal of CCR7 ligands does allow these CXCR5high naive T cells to enter follicles. Reciprocally, maintenance of CCR7 expression on activated CXCR5+ T cells is sufficient to inhibit their follicular access. This work thus extends on earlier reports (9, 10) to provide further evidence that the key changes required during T cell activation to evoke follicular homing of T cells are the up-regulation of CXCR5 and down-regulation of CCR7. This process provides a further example for how cell positioning can be precisely controlled by subtle shifts in the balance of responsiveness to chemokines emanating from adjacent zones (30).

CXCR5-deficient T cells were found previously to have a reduced ability to support early Ab responses, and it was proposed that this was secondary to a CXCR5 requirement for T cells to localize to the B/T boundary (9, 10, 44). However, these studies did not directly address whether CXCR5 deficiency affected T cell access to the B/T boundary. We considered it unlikely that it would be required because naive T cells are able to access this region despite lacking CXCR5 expression. In our transfer experiments,

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Sections were stained with anti-CD3e (blue; left panel) or anti-PD-1 (blue; right panel) together with anti-IgD (brown). T, T zone. Brown staining identifies the follicular mantle. Data are representative of four experiments (n = 10 mice). Objective magnification, ×10. C, Immunohistochemistry of sections showing the distribution of PD-1+ T cells within Peyer’s patches. Serial sections were stained, as indicated. The dashed box in the right panel highlights an area of the GC light zone that was examined at higher magnification for specific PD-1 staining of CD3 T cells. Data are representative of nine Peyer’s patches from three mice. Objective magnification, ×5 (upper panels); ×10 (lower panel). D, Flow cytometric analysis of the CCR7 and PD-1 expression levels on wild-type and CXCR5+/- OT-II T cells isolated from wild-type recipient LN on day 7 of the immune response to DEL-OVA in alum (n = 12). E, Flow cytometric analysis of the CXCR5, CCR7, and PD-1 expression levels on wild-type OT-II T cells isolated from wild-type, μMT, and CD19-deficient recipient LN on day 7 of the immune response to DEL-OVA in alum (n = 3). The frequency of transferred T cells (±SD) in each of the gates is indicated.
we confirmed that CXCR5-deficient OT-II T cells could accumulate in the outer T zone with similar efficiency to wild-type T cells. We also performed transfers of naive OT-II T cells into preimmunized hosts, and analysis 6–24 h later revealed that the cells accumulated in the splenic outer T zone near the B/T boundary (data not shown). This positioning was CXCR5 independent, but Ag (OVA) dependent, and may reflect the occurrence of long interactions between the T cells and Ag-bearing dendritic cells in this region. Whatever the explanation, these findings are consistent with the ability of the CXCR5-deficient OT-II T cells to support a normal plasma cell response at day 7. The basis for the discrepancy between our findings and previous reports showing CXCR5-deficient T cells supported slightly reduced IgG production (44) is unclear, but could reflect different requirements for interactions between T and B cells during responses to different types of Ag.

In earlier experiments, we observed that although activated T cells tend to have lower amounts of CXCR5 than B cells, they are more sensitive to low CXCL13 concentrations in chemotaxis assays (5). Our finding that CXCR5 transgenic T cells also exhibit greater dose sensitivity than B cells, including T cells that express similar amounts of CXCR5 to B cells, indicates that this difference is an intrinsic difference between T and B cells rather than being due to differences in the activation states of the cells. The finding that T and B cells differ in their PI3K and DOCK2 use when responding to lymphoid chemokines provides a possible explanation for the differences in CXCL13 chemokine sensitivity of the two cell types (45, 46).

Immunization experiments with CXCR5-deficient mice have shown that the magnitude of the GC response is reduced and the GC are mislocalized (2, 10, 47). Because CXCR5 is required for B cell homing and normal maturation of primary follicle FDCs (1, 2), it seemed likely that these deficiencies would account for the defective GC formation. However, because CXCL13 is abundantly expressed within the GC light zone and is needed for normal GC polarity (25), it also seemed likely that T cells would require CXCR5 for GC access. Surprisingly, we found that although T cell CXCR5 was needed for formation of GC responses of normal magnitude, many CXCR5-deficient T cells were able to access follicular GC. During the preparation of this manuscript, Arnold et al. (44) made very similar observations using a CXCR5-deficient bone marrow chimera approach. The basis for CXCR5-deficient T cell access to the GC is unclear, but might reflect the existence of additional GC-specific attractants. Alternatively, because activated T and B cells can form stable conjugates in which only the B cell remains motile (33), it is possible that the T cells are dragged into the GC with cognate B cell partners during formation of the GC. Given that CXCL13 is concentrated in the light zone pole of the GC (25, 48), we propose that the CXCL13-CXCR5 chemokine receptor pair contributes to directing T cells into this zone and/or retaining them there. Indeed, CXCR5 plays a role in guiding GC B cells to the light zone, whereas B cell positioning within the GC dark zone depends on CXCR4 (25). Human GC T cells have been reported to express CXCR4, but are poorly responsive to the CXCR4 ligand, SDF-1, in vitro (49). This poor responsiveness may help ensure that GC T cells favor the CXCL13-enriched light zone to the SDF-1-enriched dark zone.

IL-4 is a potent B cell growth factor, yet, although some studies have indicated that IL-4 promotes the GC response (41, 42), the extent of IL-4 expression in follicular Th cells has been unclear (7, 16, 17). Our finding that IL-4 transcripts are enriched in CXCR5highCCR7low T cells among Th cells in the 4get-OT-II and VDJ9/e5 cotransfer system is consistent with a role for IL-4 in the GC response (41, 42). Analysis of 4get reporter mice in two additional systems, transferred anti-HEL TCR7 transgenic 4get T cells in recipients immunized with HEL and 4get T cells in nontransgenic mice immunized with SRBC, again revealed that CXCR5highCCR7low T cells had abundant IL-4 transcripts (data not shown), further supporting an association between follicular Th cell differentiation and IL-4 expression. However, some studies have found limited effects of IL-4 deficiency on the GC response, suggesting differential contributions of IL-4 depending on the type of response, perhaps reflecting redundancy in some cases with IL-21, a related cytokine expressed by follicular Th cells (50, 51). IL-4 may also function in the GC to promote B cell switching to IgG1 and IgE (51), although we observed stronger switching to IgG2a than IgG1 in the OT-II and VDJ9/e5 cotransfer system (data not shown). Studies by Locksley and co-workers (52) have shown that IL-4 transcript-expressing T cells are not necessarily making IL-4 protein, and it will be important in future work to determine under what conditions GC-associated T cells produce IL-4.

PD-1 is a negative regulatory molecule that becomes up-regulated on T cells during activation (18). The PD-1 ligands, PD-L1 and PD-L2, are widely expressed, including expression on T cells, B cells, macrophages, and dendritic cells (18). Studies by Okazaki and Honjo (18) have provided evidence that PD-1 has a role in negatively regulating B cell responses, because PD-1-deficient BALB/c mice develop autoantibody-mediated dilated cardiomyopathy and PD-1-deficient B6 mice develop a systemic lupus erythematosus-like disease. The significance of PD-1 expression by GC-associated T cells is not yet clear, but it might play a role in regulating the quality of GC T cell-B cell interactions. Although minimal expression of PD-L1 and PD-L2 has been reported to be observed within GC (53), the use of more sensitive detection techniques will be required to clarify this issue. The requirement for B cells and a GC response to induce PD-1high surface expression on CXCR5+ T cells suggests that induction of PD-1 is secondary to interaction with GC B cells. Several studies have indicated that chronic exposure of CD4 and CD8 T cells to cognate Ag causes high-level PD-1 expression (18, 54). We speculate that GC T cells are chronically activated due to ongoing interactions with the numerous activated Ag-bearing B cells in the GC (21). Further studies will be needed to decipher whether PD-1 plays a negative regulatory role on follicular Th cells within the GC, such as helping prevent newly emerging autoreactive B cells from receiving T cell help.

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